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Effects of group size on maternal allocation in a colonial cooperatively breeding bird, the sociable weaver

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Resumo

Para que os indivíduos possam maximizar o seu sucesso reprodutor, a alocação de energia e de recursos na reprodução deve ser equilibrada, tendo em consideração o investimento noutras funções, como fecundidade futura e sobrevivência[1].

A alocação de conteúdos nos ovos é um mecanismo epigenético através do qual as fêmeas ajustam os recursos durante o desenvolvimento embrionário, afetando profundamente o fenótipo e *fitness* das crias[2,3]. A alocação de hormonas e carotenóides presentes nos ovos depende das condições ambientais[4] e afeta tanto o comportamento como a fisiologia das crias. A alocação de androgénios (testosterona e androstenediona (A4)) nos ovos depende da dieta alimentar e do ambiente social em que está enquadrada a mãe[5–7] e afetam positivamente o crescimento e competitividade das crias[8–18]. Mães com elevados níveis de *stress* têm maiores concentrações de corticosterona em circulação no plasma e passam-na passivamente aos seus ovos[19,20], o que pode afetar negativamente o crescimento e sobrevivência das suas crias[20]. Por fim, os carotenóides que participam em funções imunológicas importantes e afetam a cor da plumagem e do bico são apenas adquiridos através dos alimentos e a sua deposição nos ovos depende dessa disponibilidade[21–25].

Em espécies que têm reprodução cooperativa, indivíduos sexualmente maduros chamados "ajudantes" adiam o início da sua atividade reprodutora e prestam cuidados às crias de outros indivíduos. Estes sistemas são interessantes para estudar alocação maternal por diversas razões: i) as fêmeas podem prever o número de ajudantes antes da reprodução (se os ajudantes forem descendência de um ou de ambos os pais e/ou se os grupos forem estáveis durante o ano); ii) os ajudantes melhoram os cuidados das crias, nomeadamente na quantidade de comida recebida; iii) a variação dos tamanhos dos grupos entre a mesma ou diferentes fêmeas cria uma heterogenia nos cuidados que as crias recebem. Estudos anteriores sugerem que as fêmeas podem contrabalançar os custos da reprodução na presença de ajudantes, reduzindo o tamanho dos ovos e a alocação de nutrientes que sejam custosos, uma vez que os ajudantes irão compensar este menor investimento inicial trazendo alimento extra às crias logo após a eclosão dos ovos[26,27]. Deste modo, as fêmeas conseguem beneficiar da ajuda, poupando energias e/ou recursos para próximas posturas ou para a sua própria sobrevivência, permitindo-lhes maximizarem o sucesso reprodutor ao longo da vida.

Adicionalmente à reprodução cooperativa, pode também ocorrer incubação assíncrona. Esta ocorre em várias espécies de aves e carateriza-se pelos progenitores começarem a incubar antes de a postura estar completa[28]. Consequentemente, a eclosão dá-se também de forma assíncrona, do primeiro para o último ovo colocado, criando-se assim uma hierarquia entre as crias[29,30]. As crias que nascem em último lugar terão que competir com as crias mais velhas e maiores, tendo por isso menores possibilidades de sobrevivência[31]. Esta estratégia ocorre normalmente em ambientes onde a sobrevivência de posturas completas nem sempre é possível, assegurando a sobrevivência de pelo menos uma cria (hipótese da redução da ninhada)[32]. Quando as condições do meio são mais favoráveis, dando a todas as crias maiores probabilidades de sobrevivência, as fêmeas poderão compensar pelos efeitos adversos da hierarquia criada pela incubação assíncrona (hipótese do ajuste da

incubação assíncrona)[33] e depositar hormonas que potenciem o crescimento rápido das crias mais novas-

O número de ajudantes afeta positivamente as condições nas quais as crias são criadas e por isso, poderá afetar a probabilidade de sobrevivência. Por um lado, poucos ajudantes num grupo poderão não providenciar as condições necessárias para alimentar uma postura completa e as fêmeas podem enviesar a alocação de nutrientes que são custosos (carotenoides, proteínas e lípidos) para o primeiro ovo da postura, que é o que tem maior probabilidade de sobreviver. Por outro lado, um número elevado de ajudantes poderá fornecer boas condições e favorecer a sobrevivência de posturas completas. Neste caso, as fêmeas poderão compensar as diferenças de hierarquia através da deposição de androgénios nos últimos ovos da postura de modo a potenciar a competição e crescimento das crias mais novas. Contudo, a produção dos últimos ovos da postura tem maiores custos, visto estes serem produzidos depois do pico de maior exigência energética[34], sendo que as fêmeas poderão estar particularmente stressadas durante este período e, assim, transmitir passivamente maiores concentrações de corticosterona.

O tecelão social, *Philetairus socius*, é um passeriforme endémico da África Austral, com reprodução cooperativa facultativa[35]. Os indivíduos desta espécie podem reproduzir-se em pares ou assistidos por um máximo de oito ajudantes (na população estudada). Os ajudantes contribuem para a alimentação das crias e são, na sua maioria, descendentes de um ou de ambos os progenitores. Esta espécie vive em ambientes secos e imprevisíveis, onde a reprodução está ligada à ocorrência de chuvas e onde ocorre predação elevada dos ovos e das crias[36]. A esperança de vida relativamente longa e a variabilidade das condições de reprodução são favoráveis à evolução de estratégias flexíveis ao nível da alocação maternal nesta espécie.

Um estudo anterior em tecelões sociais mostrou que as fêmeas assistidas por ajudantes depositam menores níveis de androgénios e corticosterona nas gemas do primeiro ovo de cada postura comparativamente às fêmeas que se reproduzem em pares[27]. No entanto, o padrão encontrado no primeiro ovo pode não ser representativo da postura completa, pelo que o estudo dos restantes ovos da postura é essencial para o conhecimento das estratégias reprodutivas nesta espécie. Espera-se que nesta espécie a alocação maternal de nutrientes custosos e de hormonas varie com o número de ajudantes e de acordo com a sequência da postura, uma vez que a redução da ninhada é comum nesta espécie e os custos de produção dos ovos variam de acordo com a ordem de postura.

O objetivo deste estudo é, pois, investigar o efeito do número de ajudantes na alocação maternal de acordo com a sequência da postura em tecelões sociais. Para determinar o investimento total em cada ovo, 399 posturas foram pesadas durante a época reprodutiva (entre Setembro 2014 e Janeiro 2015), sendo que o tamanho do grupo e a sequência dos ovos foram determinados para 101 posturas. Para o estudo da alocação maternal a nível dos componentes dos ovos recolheram-se 36 posturas, das quais determinou-se o peso do albúmen e da gema e analisou-se os níveis de proteínas, lípidos, carotenóides e hormonas (corticosterona, testosterona e A4) presentes nas gemas de cada ovo. Destas 36 posturas, o tamanho do grupo e a sequência da postura foram determinados para 18 posturas.

Os resultados mostraram que as fêmeas diminuem o tamanho dos ovos até certo número de ajudantes (4 ajudantes). Pelo contrário, fêmeas que se reproduziram em grupos contendo mais de 4 ajudantes puseram ovos maiores, sobretudo no segundo ovo da postura, possivelmente devido ao aumento do nível de albúmen. A gema e as proteínas apenas aumentaram com o número de ajudantes e não dependem da sequência em que os ovos foram colocados. Este resultado sugere que a fêmea aumenta o conteúdo dos ovos quando se reproduz em grupos grandes porque os custos de alimentar as crias (com maiores tamanhos e consequentemente maiores exigências alimentares) é apenas suportado quando muitos ajudantes estão presentes.

Em relação à alocação dos componentes dos ovos, as proteínas, carotenóides, androgénios e corticosterona variaram segundo a ordem de postura independentemente do número de ajudantes. Fêmeas depositaram mais carotenóides e androgénios (testosterona e A4) nos primeiros ovos. Nos últimos ovos, depositaram mais proteínas e corticosterona. Este resultado está de acordo com os constrangimentos da reprodução assíncrona, mostrando que existe um maior investimento por parte das fêmeas nos primeiros ovos.

Por fim, os níveis de lípidos investidos variaram entre ovos da mesma postura de acordo com o número de ajudantes: fêmeas pertencentes a grupos com um grande número de ajudantes aumentaram as quantidades de lípidos com a sequência em que foram colocados e em grupos pequenos diminuíram com a sequência. Este resultado sugere que as fêmeas que se reproduzem em grupos grandes poderão compensar os efeitos negativos da incubação assíncrona através da deposição de mais lípidos nos últimos ovos e favorecer a sobrevivência da postura completa. Em grupos pequenos, o número de ajudantes não é muitas vezes suficiente para que todas as crias da postura sobrevivam e, por isso, as fêmeas poderão favorecer os primeiros ovos através de um investimento maior em lípidos.

Este estudo mostra a importância de se considerar a sequência da postura para se estudar o efeito do tamanho do grupo na alocação materna em espécies que se reproduzem cooperativamente.

Palavras-chave: cooperação reprodutiva, alocação maternal, incubação assíncrona, tamanho do grupo, sequência dos ovos

Abstract

In cooperatively breeding species, individuals called "helpers" assist breeders by providing care to their offspring. In these species, mothers reduce egg size and costly nutrients when assisted by helpers. This strategy may vary according to laying sequence, given the different costs of egg production and the survival probability of chicks within the clutch. Nonetheless, it is not known whether the group size affects maternal allocation according to the laying order. On one hand, when the number of helpers does not provide favourable conditions to feed the entire clutch, mothers may bias the allocation of costly nutrients in first-laid eggs and promote hierarchical differences between siblings. On the other hand, as large groups provide good rearing conditions, females may mitigate the hierarchical differences and facilitate the survival of complete broods.

In this study, I analysed the effect of group size on egg mass and egg composition (albumen and yolk mass, proteins, lipids, carotenoids, hormones) according to laying sequence in a cooperatively breeding bird, the sociable weaver (*Philetairus socius*). The results showed that females breeding in small groups laid smaller eggs whereas in groups with more than 4 helpers, females produced bigger eggs (particularly the second eggs), possibly due to an increase in albumen mass. Both yolk and proteins increased with group size and did not depend on the laying sequence. In addition, I found an effect of laying sequence on egg composition with first-laid eggs having higher levels of carotenoids and androgens and later-laid eggs more proteins and corticosterone. Lastly, in big groups, the levels of lipids increased with laying order whereas in small groups they decreased. This study clarifies the maternal allocation strategies in this species and shows the importance of considering laying order when investigating the effect of group size on female allocation in cooperative breeders.

Keywords: cooperative breeding, maternal allocation, hatching asynchrony, group size, laying order

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Introduction

Life-history theory assumes that reproduction is costly and influenced by trade-offs between the allocation of energy and/or resources into reproduction or other functions such as future fecundity and survival. Therefore, individuals should balance their investment in reproduction in a way that maximize lifetime reproductive success[1].

Studies on the costs of reproduction have focused on the consequences in terms of survival and fecundity[37], as well as the underlying mechanisms. These mechanisms range broadly from the allocation of limited resources to the embryo[38] to increased likelihood of predation by decreasing locomotor performance (e.g. increased body mass of the pregnant female[39]) or increased susceptibility to parasitism, disease[40] or oxidative stress[41].

In oviparous species, eggs contain all the substances needed to produce fully functioning individuals and their production was for many years believed to have minor costs for females comparatively to the costs of incubation and chicks rearing. Nevertheless, growing evidence has been gathered to support the idea that eggs formation is costly both in terms of energy and nutrient requirements (reviewed in [42]). For example, in some species, the metabolic rates increase during egg formation (e.g. *Parus major*[43], *Sturnus vulgaris*[44]) suggesting an increase in energetic demand during egg production. Regarding nutrition requirements, some studies have reported muscle mass losses during the formation of eggs[45,46]. Studies involving food supplementation also supported the idea of nutrition requirements during egg formation, as females submitted to food supplemented treatments laid bigger eggs and larger clutches[47–49].

Mother's reproductive strategies in the current breeding attempt depend upon environmental conditions such as food availability, breeding density, and social interactions with mates or competitors, as well as mother's condition, survival prospects, past reproduction or genetic background[50]. Zebra finch, *Taeniopygia guttata*, females lay larger clutches when mated with more attractive males[51]. When environmental conditions are extremely poor, females may skip reproduction and save energy for future reproductive attempts which is the case of red-footed booby, *Sula sula,* that is more likely to skip reproduction in El-Niño years[52].

Allocation of contents into eggs is an important mechanism for the development of embryos as they are completely dependent on these resources that profoundly affect the offspring's phenotype and fitness[2,3]. This allocation is an epigenetic mechanism in which females adjust the developmental resources for offspring development depending upon environmental conditions.

Females can allocate different levels of costly nutrients such as lipids, proteins and carotenoids[53]. Lipids are the main constituents of yolk apart from water and provide most of the energy during growth and development[54]. Proteins are the third main constituent of yolk and a rich source of amino acids, phosphate and carbohydrate and play a role in the transport of lipids, vitamins and mineral ions from mother to eggs, which are essential for embryonic development[55]. Carotenoids are fat-soluble pigments exclusively acquired through food.

Carotenoids have an immune function in both mother and embryos[21–23] (see more roles in Table 1) and vary with food availability[56].

Table 1. Effects of yolk hormones and carotenoids on offspring phenotype.

Yolk contents		Offspring phenotype		
		Increases	**Decreases**	**Inconsistent effect**
Hormones	**Androgens**: Testosterone and A4	Dominance/competitive -ness (begging rate)[8–13], growth[8,13–18] and metabolic rate[57]	Immune response[15,58–60]	Hatching time[12,14,57,61,62] and survival[9,12,16,63]
	Glucocorticosteroid: Corticosterone	Hormonal stress response[64]	Hatching size, growth and survival[20]	Begging rate[65]
Antioxidant	Carotenoids	Immune functions [21–23], coloration of plumage[24] and gape or beak[25]		Protection against ROS[66]

Yolk contents are indicated in the first column, positive and negative effects on offspring trait are shown in the second and third columns, respectively and cases where either positive or negative effects are observed among species are shown in the last column. Abbreviations: ROS - Reactive oxygen species, A4 - Androstenedione

Hormones are also an important component of yolk content. They are chemical messengers that bind to specific receptors in their target tissues and can directly regulate gene activity or change the physiological state of cells[50]. It is yet unknown whether the transfer of hormones to eggs may be costly to breeding females. Production of hormones is unlikely to be energetically costly because steroids are produced from the abundant cholesterol[67], but females may suffer costs when having elevated levels of hormones in circulation, although convincing studies are currently needed[68]. Mothers can allocate different levels of hormonal contents such as androgens and corticosterone into eggs depending upon environmental variations[4] with implications on offspring's physiology and behaviour (see main roles in Table 1). Females experiencing stressful pre-breeding environment deposit passively more glucocorticoid such as corticosterone[19,20]. Androgens (testosterone and androstenedione (A4)) are actively transferred by the mother depending on pre-breeding conditions such as diet quality and social environment[5–7]. In addition, yolk androgens levels modulate sibling growth and competition between siblings (Table 1) and affect brood hierarchies depending on environmental conditions (e.g. food availability).

Hatching asynchrony is widely spread across birds' species and occurs when parents start incubation before clutch completion[28]. This asynchrony which follows laying order produces a brood hierarchy among siblings[29,30]. The later-hatched chick will have to compete with older and bigger siblings and consequently will have a lower likelihood to survive[31]. In environments with limited food supply during nestling, females may facilitate brood reduction through allocation of high levels of androgens in first-laid eggs – brood reduction hypothesis[32]. In environments where food availability is sufficient for rearing the whole

brood, females may mitigate the disadvantage of being a later-hatching chicks and allocate androgens into later-laid eggs to enhance growth, enabling them to compete with the older siblings and adjust the survival of these younger chicks – hatching asynchrony adjustment hypothesis[33].

In cooperative breeding systems, sexually mature individuals called "helpers" forgo independent reproduction but instead help other individuals to breed. This helping behaviour is widespread among *taxa*, including insects[69], birds[70], fish[71] and mammals[72].

Cooperative breeding species is an interesting model to study maternal allocation for several reasons: i) females may predict the number of helpers before reproduction (for example if helpers are generally offspring of one or both breeders and/or groups are stable throughout the year), ii) helpers improve raising conditions and iii) variation on group size among and within females creates ecological heterogeneity in rearing conditions which is expected to favour variation in maternal allocation[73].

In the presence of helpers, breeding females can either invest more in eggs to take advantage of the good conditions (i.e. more helpers) or counterbalance the costs of egg production by reducing egg size because helpers will compensate during nestling by bringing additional food[73]. To date, most studies corroborate the "load-lightening" hypothesis (but see [74]) egg size is reduced in the presence of helpers in cichlid fishes[75], superb fairy-wrens[26], carrion crows[76], southern lapwings[77] and sociable weavers.Nevertheless, egg size is a crude measure of female energetic investment in reproduction and analysis of egg contents provides a more detailed interpretation of the extent in investment and its fitness consequences for mothers and offspring. In the cooperatively breeding superb fairy-wren *Malurus cyaneus*, it was found that in addition to the reduction in egg size when females bred in groups, eggs had also lower nutritional contents (12% less lipids and 13% less proteins than those laid by females in pairs)[26]. In sociable weavers, *Philetairus socius*, females assisted by helpers had lower yolk hormonal levels of androgens (testosterone and A4) and corticosterone in their first laid eggs, but no differences were found in yolk mass and carotenoids concentration[27]. Maternal allocation of these components is known to vary within the laying sequence[78], although none of these studies paid attention to the variation of yolk contents within the laying sequence according to the number of helpers.

The aim of this study is to investigate the effect of the number of helpers on female allocation according to laying sequence in the sociable weaver, a relatively long-lived cooperatively breeding monogamous passerine. They live in a dry, unpredictable environment where reproduction is linked to rainfall patterns and where predation of eggs and nestlings is high[36]. This unpredictability in reproductive success and relatively long lifespan are expected to favour the evolution of maternal allocation flexibility in this species[27].

Egg mass and egg composition (albumen and yolk mass, proteins, lipids, carotenoids, corticosterone and androgens) were analysed according to laying order and group size.

Brood reduction is common in this species and the first-laid eggs have the highest probability of survival[35]. The previous study showed that sociable weavers' females bias allocation of yolk mass and carotenoids which is higher in the first eggs of the clutch[78] and may ensure the survival of at least the chick hatching from this first egg. Nonetheless, the increase of egg size through deposition of albumen in the following eggs[78] may be less costly

for females comparatively to the costs of yolk and antioxidants and may, to some extent, mitigate brood hierarchies when environmental conditions are favourable[79]. In addition, the number of helpers may influence chicks' survival probability or competitive ability between siblings and consequently maternal allocation strategies should vary within the clutch according to group size. When the number of helpers does not provide favourable conditions to feed the entire clutch, mothers may bias the allocation of costly nutrients in first-laid eggs and promote hierarchical differences between siblings. On the other hand, as large groups provide good rearing conditions, females in large groups may mitigate the hierarchical differences and facilitate the survival of complete broods through deposition of androgens that enhance competitiveness and growth on later-laid eggs (Table 1). Therefore, I expected that females in larger groups favour the survival of the complete brood and mitigate hierarchical differences through deposition of androgens on later-laid eggs, whereas females in smaller groups should deposit costly egg components such as yolk and its constituents (carotenoids, proteins and lipids) in first-laid eggs considering that they have higher chance of survival[35]. Moreover, the yolk of later-laid egg is more costly to produce because it is produced during the energy peak demand[34] and thus females are expected to be more stressed during this period and then transmit passively more yolk corticosterone on later-laid eggs.

Methods

Study Species

The sociable weaver is a facultative cooperative breeding passerine endemic to the semi-arid savannahs of southern Africa[80].

This species builds communal nests, usually on horizontal branches of acacia trees, containing several independent chambers used for breeding and roosting. Nests are occupied all year round and buffer environmental extremes[78]. Colonies sizes range from less than ten to several hundred individuals[81].

Sociable weavers are sexually monomorphic, monogamous and can breed in pairs or assisted by up to 8 helpers (average group size for breeding season 2014 is 4.172±1.683)[36]. Helpers are usually offspring of one or both breeders, although up to 7% of helpers are more distant relatives or unrelated[82]. Females and males help in their first year, but older or unrelated helpers are usually males[83]. Helpers assist the breeders mostly by feeding the nestlings. Females usually lay 3-4 eggs (one per day) and can have several broods per breeding season. If egg predation occurs, a single female may produce up to 13 clutches in one season (unpublished data). Both breeders incubate over 15 days and the nestling period occurs along 21-24 days[80].

Field Methods

Study site

Field work was conducted between August and December of 2014 at Benfontein Nature Reserve near Kimberley in Northern Cape province of South Africa (28º52' S, 24º50' E). This area is semiarid, experiencing low and unpredictable rainfall, with most of the precipitation falling during the summer time (September-April).

Capture of individuals before breeding season

Since 1999, colonies are annually captured by setting a mist nest around the colony before dawn, when birds are roosting inside. Before the breeding season has started, between late August and early September, individuals from 14 colonies were caught using mist nets. Eggs from six of these colonies were collected for the analysis of yolk mass and egg contents, although all the 14 colonies were used for the analysis of egg mass in this study (Table S1). Half of the sampled colonies were protected against snakes with wrapping cling plastic around the tree trunk to avoid the predation of eggs and ensure enough data to collect (Table S1). Individuals were processed and released on the site of the captures. Unringed birds were ringed with a colour ring combination and a unique numbered aluminium ring. Body mass and tarsus length were systematically measured. Blood samples were also taken for genetic sexing and determination of parentage. Subcutaneous passive integrated transponders (PITs)

for individual identification were implanted dorsally in birds from four colonies (Table S1). The number of individuals caught in the nets and birds that escaped during the captures was used as colony size.

Sample collection

Colonies were routinely checked for eggs every 5 days. Each chamber of the colony was inspected for the presence of eggs with a mirror and a flashlight from a vehicle rooftop parked beneath the colony. After the first egg was found, indicating the beginning of the breeding season, 6 colonies were visited daily during September and October until I collected 36 complete clutches. All eggs collected for the yolk mass and contents analysis (n=106 eggs from 36 clutches) were daily marked with a soft blunt pencil to assess the laying order (one egg is laid per day). Two days after the first egg of the clutch was found, the eggs were weighted (Digital Pesola Balance, accurate to 0.001g) and the clutches were collected and stored at -20ºC until further laboratory analysis. In the next day, the chambers where I collected the eggs were checked again to search for a possible fourth egg. In rare cases, when a fourth egg was laid, it was weighted and stored as described above.

Regarding the egg mass analysis, in addition to the 36 clutches weighted before, 363 clutches were weighted and marked between September and January as described before. When the first egg was found, the chambers were subsequently visited the following 3 days to mark the laying order of complete clutches. In chambers where two or more eggs were simultaneously found, the information on laying order was incomplete (the chamber were inspected to mark the remaining eggs of the clutch) or not determined (the chamber was inspected when the female had already laid a complete clutch).

The incubation period begins with the first or second egg[84] as a result, the eggs were incubated differentially according to the laying position. However, the eggs sampled in this study were incubated for a maximum of 2 days (the case of the first-laid egg) before being collected, therefore, the yolk-to-embryo transfer is expected to be minimal at this extent[6,78,85].

Differences in incubation time within the clutch could lead to differences in egg mass loss. Although, in sociable weavers, eggs decrease in mass on average 0.025±0.017 g per day (based on 114 eggs weighted on successive days early in the incubation period), which is insufficient to account for the difference through laying sequence[78].

Breeders' identification

I identified all the individuals who visited the chamber during incubation, before the sample collection on the third day, to ensure I had the identification of one or both breeders (four clutches with one breeder detected, 28 clutches with both breeders out of the 36 clutches collected). I identified these individuals using video recordings (Sony Handycam HD), pit tags and direct observations. The cameras recorded the entrance of the chamber for a minimum of 1h per day during 1-3 days. The direct observations were conducted from a hide placed 2-5m from a colony for a minimum of 1h a day over 1-2 days. Individuals were identified in the recordings and direct observations by their colour combination when

entering or leaving the chamber. Antennas that detected the bird's PIT tag codes were positioned with wires at the entrance of the nest in order to detect birds' entrance and exit, this information was extracted after 3 days.

There is no evidence of extra-paternity cases in this species[82,86], so I considered that the individuals I have identified during the first attempt and that match the breeders obtained through genotyping analysis from the blood sample collected from the 9-day-old chicks of second breeding attempt were effectively the parents from the first attempt.

Genotyping analyses were conducted at the University of Sheffield (UK). Sex was determined by amplification of chromo-helicase-DNA-binding genes located on the W and Z sex chromosomes using the P2 and P8 universal primers[87]. To distinguish between breeders and helpers, 17 microsatellite loci were used to determinate parentage (PS1-GCSW15, GCSW47, INDIGO40, TG22-001, PS2-GCSW35, INDIGO41, Ppi2-Gga, TG01-148, WBSW9, PS3 GCSW13, INDIGO29, CAM1, CAM15, PS4-Ase18, GCSW31, GCSW57, TG07-022)[87–93].

GenePop v.4.0.1[94] was used to test for conformity to Hardy–Weinberg equilibrium for each locus. Microsatellite GCSW57 was excluded for further analysis because it was not in conformity to this equilibrium.

I used Colony v2.0.3.8[95] to assign each juvenile to a most likely father and mother by full-likelihood method. As the same group of individuals own more than one chamber within the same colony and alternate chambers between attempts, I used all the genotypes from all adult birds ever genotyped (1221 females and 1280 males) and offspring genotypes from the 7 colonies where I collected the eggs (252 chicks) in order to identify the alternative chambers. Marker typing error was set to 1% and the proportion of candidate parents genotyped was set to 75% to include the possibility of an unknown bird being the parent. I assigned parentage when parentage probability was one.

Group size determination

When the same parents were identified for the first and second attempts, I identified the group composition during the nestling period of the second attempt when helpers were actively feeding the chicks. The breeding group is stable during the breeding season[36] and therefore, after removing the eggs from the first attempt, I assumed that the group will not change until the next attempt. Furthermore, after removing the clutch, the females laid again after a short period of time (average of ≈ 11 days) and, therefore, it is unlikely that group composition has changed. An individual was considered part of the group if visited the chamber in more than one day and contributed with more than 1% for the total visits of the nest chamber. These visits were observed by cameras and direct observations for at least 1h30min a day over 3-8 days of nestling period, following the same procedures as described before.

Yolk content analysis

Yolk mass, albumen mass, proteins, lipids, carotenoids and hormones (corticosterone, testosterone and A4) were measured for 108 eggs from 36 clutches.

Yolk and shell were separated from albumen while defrosting and weighed to the nearest 0.0001 g. Yolk was homogenized and kept at -20ºC until analysis. Albumen mass was determined by subtracting yolk and shell mass from egg mass.

For proteins and lipids analysis, the yolk was dried at 60ºC for about 24-48 hours. The nitrogen concentration of the samples was determined with an elemental analyser Thermo-Finnigan, Flash EA 1112 Series. Protein content was calculated from the nitrogen values using a conversion factor of 6.25[96]. Lipids were extracted using chloroform and measured gravimetrically[97].

Carotenoids concentration was determined by colorimetry[98,99]. The wet yolk was diluted in acetone and optic density (OD) was obtained at 450 nm using microplate photometer (Victor^3 1420 Multilabel Plate Reader, Perkin-Elmer).

Hormones were quantified by radioimmunoassay, based on the antigen-antibody reaction[100]. Corticosterone assays used the antibody "anti-11-HS-corticosterone" supplied by P.A.R.IS. (France). For testosterone, it was used the antibody "Anti-testosterone" provided by Dr. Picaper (CHU La Source, Orléans, France) and A4 used "anti-androstenedione" by Sigma (US). Cross-reactions of corticosterone antiserum were as follows: cortisone (53 %), 20α-hydroxyprogesterone (2.5 %), cortisol (2 %), progesterone (1.3 %), Δ4-pregnen-21-ol-3,20-dione (0.5 %), aldosterone (0.2 %), 17α-hydroprogesterone (0.1%), 1,3,5(10)-estratrien-3,17-diol (0.1 %), 20β-hydroxyprogesterone (<0.03 %).

Detailed methods are described in the supplementary material (Appendix 1).

Sample size

Egg mass analysis

For the analysis of egg mass, we identified 56 groups (173 clutches between 1st to 5th attempts) of the total 399 clutches weighted during the studied breeding season (September 2014 to January 2015). The complete laying order was assessed for 53 clutches and incomplete laying order for 48 clutches out of 173 clutches, providing a final sample of 101 clutches for which I have complete or incomplete laying sequence. For these clutches, the clutch size varied between 2 (n=6), 3 (n=61) and 4 (n=34) eggs. Fifteen of the clutches were from the non-protected colonies while 86 clutches were from protected colonies. The mother identification was conducted for 92 clutches.

Egg composition analysis

The clutch size of the 36 clutches collected varies between 2 (n=4), 3 (n=28) and 4 (n=4) eggs. As a result, clutches of two and fourth eggs were excluded as there was an insufficient sample to test a possible effect of clutch size in relation to laying order and group size. The identity of both parents was determined for 20 of these 28 clutches while mother's identification was confirmed for 21 clutches. Group composition was determined for 19 clutches out of the 28 clutches (one breeding pair did not reproduce a second time and eight clutches did not have enough information about breeders or group composition to be included). One group was found to re-lay the second attempt 52 days after the first and then excluded from the analysis due to the longer time to relay comparatively to the remaining clutches. Thus, the final sample size was 18 clutches where group size was considered in the minimal model. Out of the 18 clutches, the identities of both parents were confirmed for 16 clutches and mother's identification for 17 clutches.

Statistical analysis

The aim of this project was to study if the group size affects the maternal allocation. To test this, Linear Mixed Models (LMM) were performed in R software[101] using the packages "nlme"[102] and "lsmeans"[103]. The final models were obtained by sequentially excluding explanatory variables with P-value > 0.1 using backwards stepwise approach. The minimal model provided the P-values for significant terms whereas P-values for non-significant terms were obtained by reintroducing each non-significant variable into the minimal model[104]. Residuals of the models were visually inspected (QQ plots, fitted versus residual plots and histograms) to verify assumptions of the linear mixed models.

Egg mass analysis

For the analysis of egg mass, I tested the following independent variables: laying order within the clutch (first to fourth egg as a factor variable), clutch size (2 to 4), the number of breeding attempt (1 to 5th time), group size (number of breeders and helpers, from 2 to 10 individuals), quadratic term of group size, the protection/non-protection against the snakes, colony size, rainfall, laying date (number of days after the first clutch collected have been found), mother's mass (g) and tarsus length (mm) and the interaction laying order × group size and laying order × group size^2. Mother's mass and tarsus length were included together to estimate the body condition[105]. The variable group size was maintained in the minimal model if the P-value of the quadratic term of group size or the interaction laying order × group size^2 were <0.1. To account the non-independence of the eggs within clutches, different attempts from the same mother and chambers within colonies, I included "clutch ID" nested in "chamber" nested in "colony" as random factors.

Egg composition analysis

I tested both absolute and relative levels of yolk contents, as the total amount of yolk contents will give information on maternal investment while the concentration will be more meaningful for offspring fitness. I used yolk mass, albumen mass, concentration and absolute contents of yolk proteins, lipids, carotenoids, corticosterone, testosterone and A4, as dependent variables. The explanatory variables were laying order within the clutch (1st to 3rd egg as a factor variable), group size (from 2 to 8 individuals), quadratic term of group size, laying date (days), mother's mass (g) and tarsus length (mm) and the interaction laying order × group size. The variable group size was kept in the minimal model if the P-value of the quadratic term of group size or interaction laying order × group size^2 were <0.1. I included "chamber" nested in "colony" as random factors.

Considering the relatively small sample size and to avoid over-parameterising the models, I did a preliminary analysis where I tested the least important variables (laying date, mother's length of tarsus and mass, mother's minimum age) separately, except the mother's tarsus and mass that were tested together to estimate condition[105]. I kept these variables for the main analysis when they were significant ($p<0.05$). Mother's minimum age was defined as the number of years since the year of the first capture until 2014. Minimum age is close to real age for a large proportion of birds[86] and hence, I included all ages in the same variable "Mother's minimum age". Colony size (number of individuals in the colony) and the binary variable protected/non-protected colony were removed from the analysis as 80% of the samples were collected from the three biggest and protected colonies (between 80 and 120 individuals), hence these variables did not present enough variation to be included in the analysis.

Since only 2 of the identified groups were attended by the breeding pair only, I did not test the effect of group type (with or without helpers) but only the group size (number of helpers plus the breeding pair) to test the possible effect of helpers on egg mass, yolk mass and yolk contents.

To test the possible dependency between the egg mass, yolk mass, albumen mass, and relative and absolute levels of yolk contents, relations were inspected using linear mixed models using random factors "chamber" nested in "colony". For this analysis, I included all the 36 clutches collected.

Results

❖ Egg mass

Egg mass varied from 2.045 g and 3.160 g. Egg mass varied significantly in a positive quadratic way with the number of helpers according to the laying sequence within the clutch (interaction Group size^2*Laying order: F=5.207, P=0.002; Table 2). The quadratic effect was more pronounced for the second eggs (estimate=0.003±0.001; Table 2; Figure 1). In addition, egg mass tended to decrease with clutch size (F=3.043, P=0.088, estimate=−0.045±0.026). The egg mass was not affected by laying date, number of clutches attempted before, protection of the colonies, rainfall, mother's condition, mother minimum age and colony size (all P>0.329). Yolk mass and albumen mass are both dependent of egg mass (P<0.002; Table S2).

Table 2. Factors affecting the egg mass

Explanatory terms	F	P	Estimates	SE	*df*
Intercept			**2.885**	**0.169**	**120**
Laying order	**8.595**	**<0.001**			
2			**0.033**	**0.030**	**120**
3			**0.135**	**0.028**	**120**
4			**0.061**	**0.040**	**120**
Clutch size	**3.043**	**0.088**	**-0.045**	**0.026**	**48**
Group size	**2.961**	**0.093**	**-0.105**	**0.061**	**39**
Group size^2	**2.688**	**0.109**	**0.010**	**0.006**	**39**
Group size^2 × Laying order	**5.207**	**0.002**			
Group size^2 × 2			**0.003**	**0.001**	**120**
Group size^2 × 3			**-0.001**	**0.001**	**120**
Group size^2 × 4			**-0.001**	**0.001**	**120**
Laying date	0.424	0.518			
Breeding attempt	0.001	0.974			
Protected/Non-protected	1.065	0.329			
Rainfall	0.617	0.436			
Mother's mass	0.844	0.368			
Mother's tarsus	0.501	0.486			
Mother's min. age	0.002	0.968			
Colony size	0.954	0.354			

Estimates and SE are given for explanatory terms included in the minimal model (bold characters)

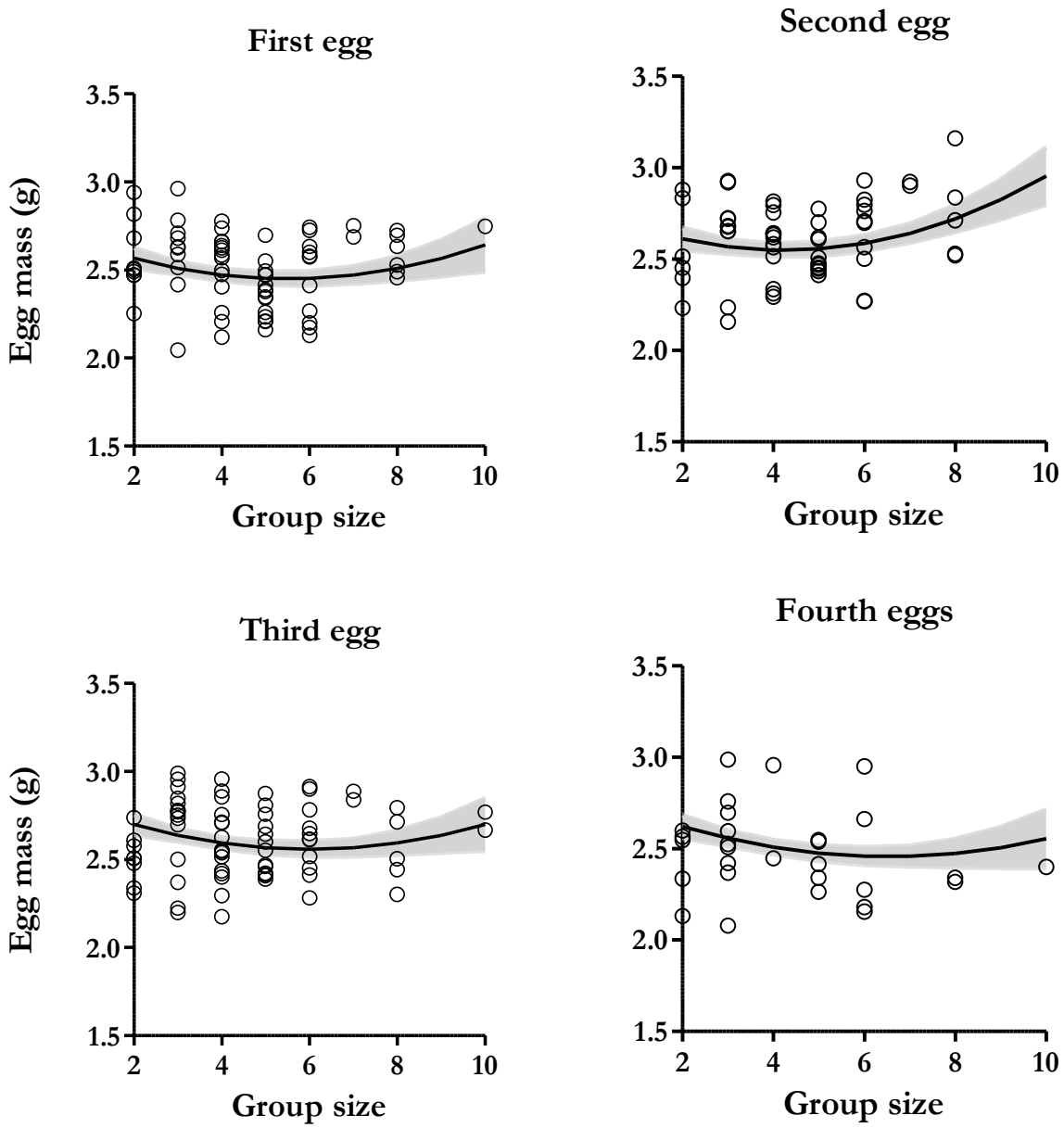

Figure 1. Effect of the interaction between group size and laying order on egg mass. The full-filled line indicates predicted values from the linear mixed models and grey area correspond to ± SE values. Dots are the values of egg mass per egg ($N_{first\ eggs}$=69, $N_{second\ eggs}$=57, $N_{third\ eggs}$=72; $N_{fourth\ eggs}$=29).

❖ Albumen mass

Albumen mass varied between 1.238 g and 2.355 g and increased significantly with the number of helpers depending on laying order within the clutch (F=6.658, P=0.005), this effect being significantly more pronounced on second eggs (estimate=0.064±0.018; Table 3; Figure 2). Additionally, albumen mass tended to increase with mother's mass and decrease with mother's tarsus length but these were below the significance threshold (Table 3). Mother's minimum age and laying date were not significant and not included in model selection (P>0.084).

Table 3. Factors affecting the albumen mass

Explanatory terms	F	P	Estimates	SE	*df*
Intercept			**2.680**	**1.332**	**23**
Mother's tarsus	**4.321**	**0.083**	**-0.137**	**0.070**	**6**
Mother's mass	**5.568**	**0.056**	**0.080**	**0.036**	**6**
Laying order	**2.659**	**0.091**			
2			**-0.160**	**0.086**	**23**
3			**0.020**	**0.086**	**23**
Group size	**0.408**	**0.547**	**0.015**	**0.024**	**6**
Group size × Laying order	**6.658**	**0.005**			
Group size × 2			**0.064**	**0.018**	**23**
Group size × 3			**0.016**	**0.018**	**23**
Group size^2	0.078	0.791			

Estimates and SE are given for explanatory terms included in the minimal model (bold characters)

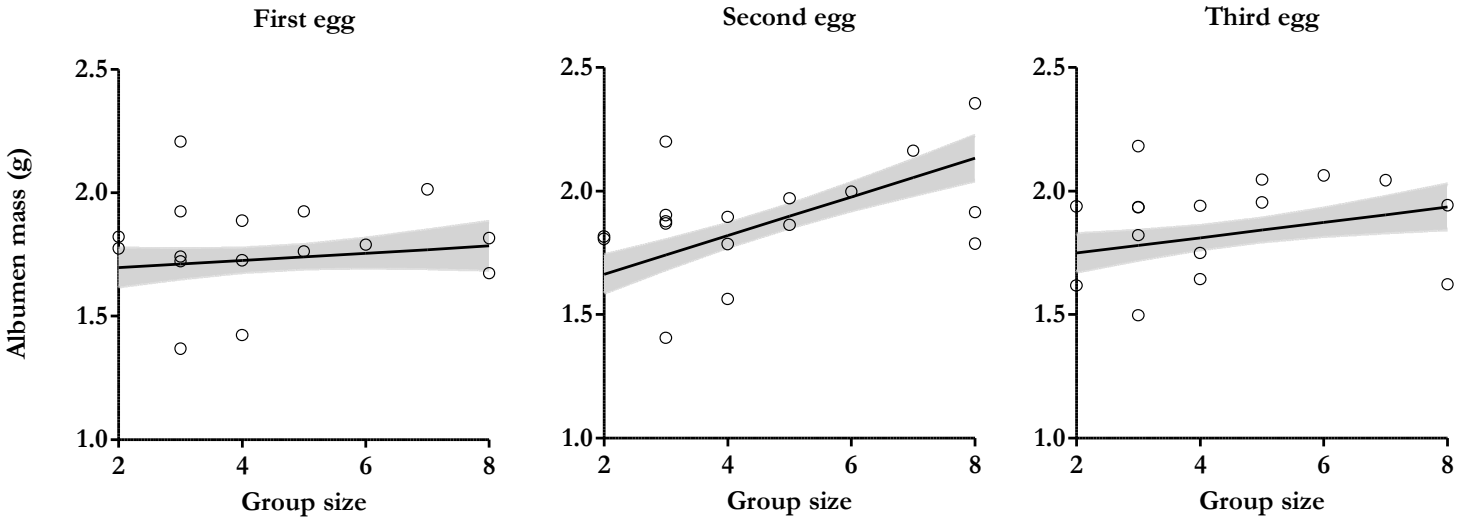

Figure 2. Effect of the interaction between group size and laying order on albumen mass. This effect is more pronounce on second eggs. The full-filled line indicates predicted values from the linear mixed models and grey area correspond to ± SE values. Dots are the values of albumen mass per egg ($N_{first\ eggs}$=16, $N_{second\ eggs}$=17, $N_{third\ eggs}$=16)

❖ Yolk mass

The wet yolk mass ranged between 0.452 g and 0.800 g. Yolk mass varied in a negative quadratic way with the number of helpers, but this is below significance (estimate=−0.005±0.002, F=4.091, P=0.068; Table 4). When the quadratic effect was removed from the minimal model, the positive linear effect of the number of helpers on yolk mass was significant (estimate=0.018±0.005, F=17.163, P=0.001; Figure 3). Yolk mass was not affected by laying order within clutches (F=2.428, P=0.104; Table 4). The condition of female, mother's minimum age and laying date were not significant and hence not included in model selection (P>0.614).

Table 4. Factors affecting the fresh yolk mass

Explanatory terms	F	P	Estimates	SE	*df*
Intercept			**0.434**	**0.056**	**34**
Group size	**7.536**	**0.019**	**0.064**	**0.024**	**11**
Group size^2	**4.091**	**0.068**	**-0.005**	**0.002**	**11**
Laying order	2.428	0.104			

Estimates and SE are given for explanatory terms included in the minimal model (bold characters)

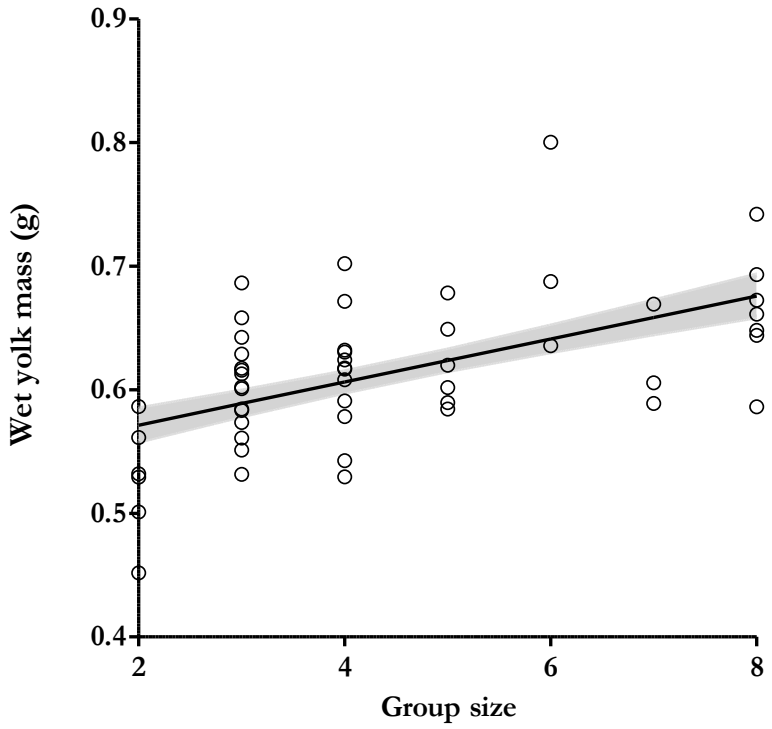

Figure 3. Relationship between wet yolk mass and breeding group size. The full-filled line indicates predicted values from the linear mixed models and grey area correspond to ± SE values. Dots are the values of yolk mass per egg (N=52). The linear effect of group size was estimated removing the quadratic term of group size from the minimal model.

❖ Yolk proteins

The absolute level of proteins ranged between 0.155 g and 0.285 g and the concentration ranged between 30.47% and 38.44%. The absolute level of proteins significantly increased with laying sequence ($F=4.478$, $P=0.020$; Table 5a), with the third egg having more quantity of proteins (Figure 5). The number of helpers had a marginally non-significant effect on mass of proteins in a quadratic negative way (estimate=-0.002 ± 0.001, $F=4.445$, $P=0.059$; Table 5a). When removing the quadratic effect of group size from the minimal model, the linear effect of group size was positive and significant (estimate=0.007 ± 0.002, $F=16.782$, $P=0.002$; Figure 4). Concentration of proteins tended to increase in third eggs ($F=2.537$, $P=0.089$; Table 5b). Group size had no effect on the concentration of proteins (linear and quadratic effect $P>0.355$). Mother's condition, minimum age and laying date were not significant and hence did not enter in model selection (all $P>0.081$).

Table 5. Factors affecting the absolute (a) and relative (b) level of yolk proteins

Explanatory terms	F	P	Estimates	SE	*df*
a) Absolute proteins					
Intercept			**0.137**	**0.021**	**31**
Laying order	**4.478**	**0.020**			
2			**-0.006**	**0.007**	**31**
3			**0.014**	**0.007**	**31**
Group size	**7.946**	**0.017**	**0.026**	**0.009**	**11**
Group size^2	**4.445**	**0.059**	**-0.002**	**0.001**	**11**
b) Concentration proteins					
Intercept			**33.705**	**0.332**	**49**
Laying order	**2.537**	**0.089**			
2			**0.318**	**0.415**	**49**
3			**0.929**	**0.419**	**49**
Group size	0.037	0.851			
Group size^2	0.924	0.355			

Estimates and SE are given for explanatory terms included in the minimal model (bold characters)

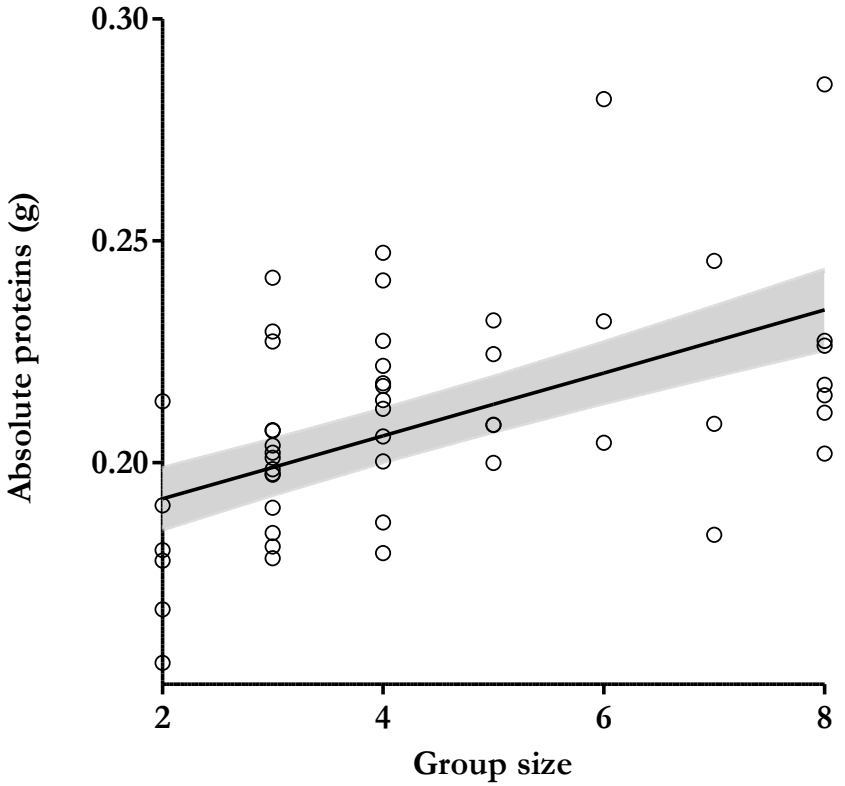

Figure 4. The linear effect of group size on absolute proteins. The linear effect of group size was estimated removing the quadratic term of group size from the minimal model. The full-filled line indicates predicted values from the linear mixed models and grey area correspond to ± SE values. Dots are the values of absolute protein per egg (N=51).

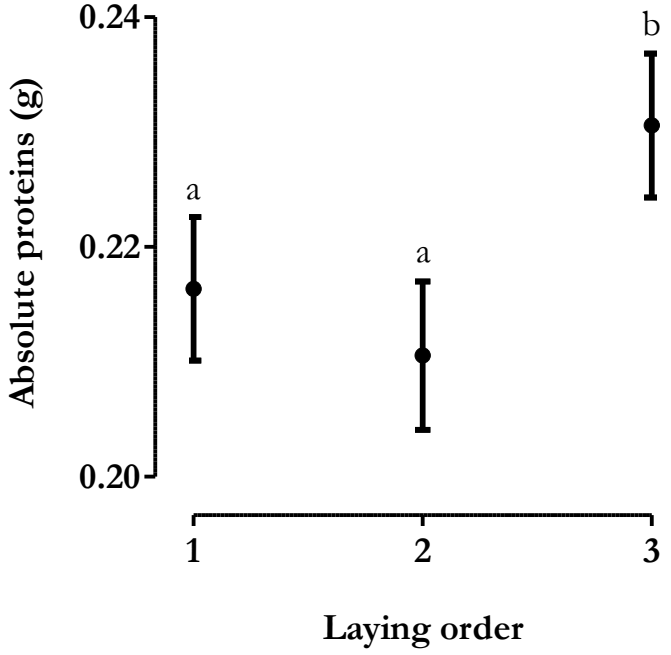

Figure 5. Absolute proteins according to laying order. The Data presented are predicted means±SE.

❖ Yolk lipids

The absolute level of lipids varied between 0.082 g and 0.597 g and the concentration varied between 15.48% and 92.56%. Mass of lipids and concentration changed significantly with the number of helpers according to the laying order within the clutch (absolute level: F=7.046, P=0.003; relative level: F=6.559, P=0.005; Table 6; Figure 6). Absolute and concentration of lipids decreased with the number of helpers on first eggs but increased on second and third eggs within the clutches. Mother's condition, min. age and laying date were not significant and hence did not enter in model selection (all P>0.0945).

Table 6. Factors affecting the absolute (a) and relative (b) level of yolk lipids

Explanatory terms	F	P	Estimates	SE	*df*
a) Absolute lipids					
Intercept			**0.401**	**0.060**	**27**
Laying order	**5.631**	**0.009**			
2			**-0.169**	**0.074**	**27**
3			**-0.243**	**0.074**	**27**
Group size	**2.321**	**0.154**	**-0.019**	**0.012**	**12**
Laying order × Group size	**7.046**	**0.003**			
2 × Group size			**0.042**	**0.015**	**27**
3 × Group size			**0.056**	**0.015**	**27**
Group size^2	2.210	0.165			
b) Concentration lipids					
Intercept			**71.564**	**9.733**	**27**
Laying order	**5.522**	**0.010**			
2			**-29.351**	**12.559**	**27**
3			**-40.638**	**12.515**	**27**
Group size	**4.879**	**0.047**	**-4.384**	**1.986**	**12**
Laying order × Group size	**6.559**	**0.005**			
2 × Group size			**7.413**	**2.525**	**27**
3 × Group size			**8.786**	**2.608**	**27**
Group size^2	1.381	0.265			

Estimates and SE are given for explanatory terms included in the minimal model (bold characters)

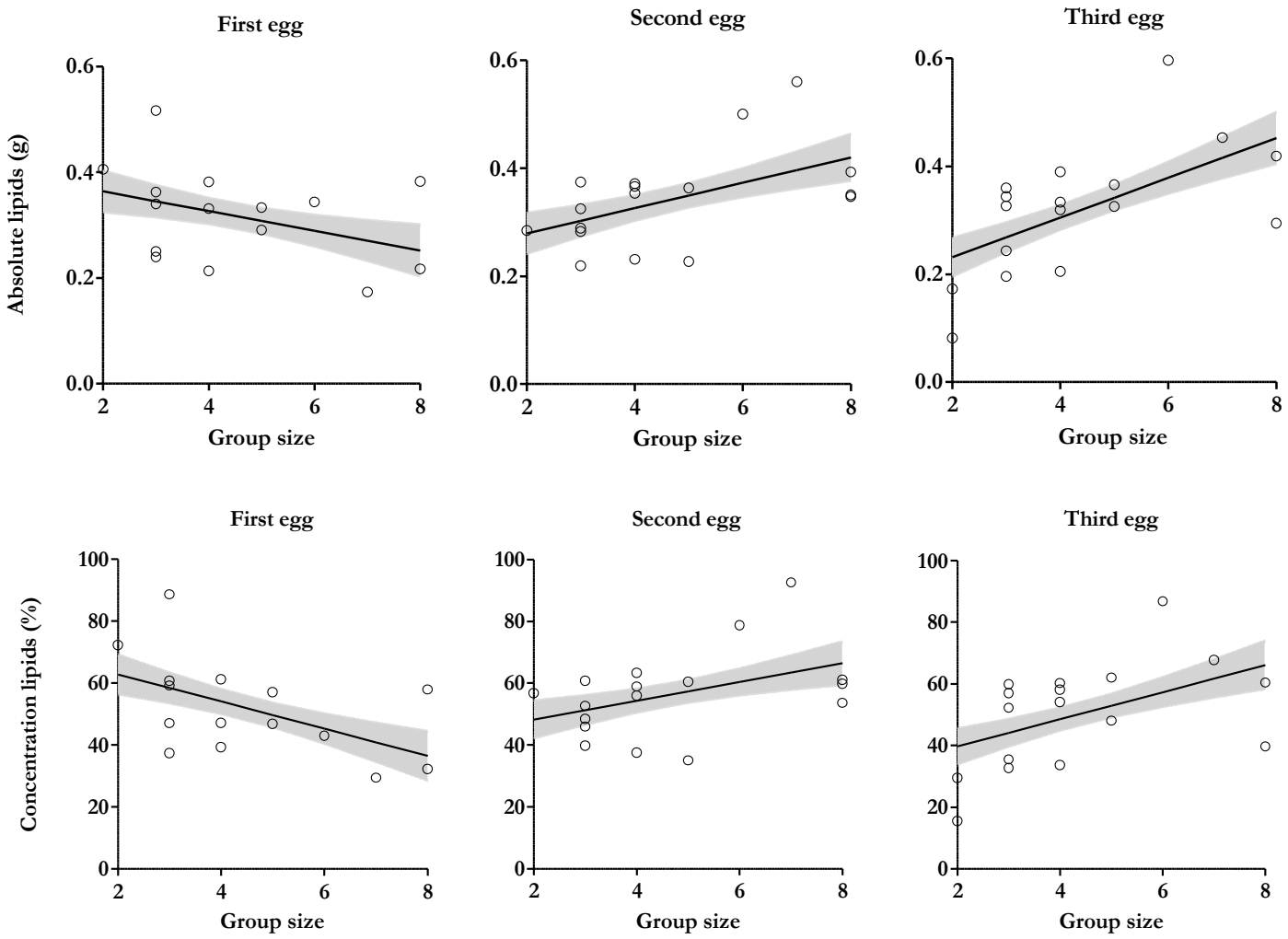

Figure 6. Effect of the interaction between group size and laying order on absolute and concentration lipids. Absolute and concentration decreased with group size on first eggs but increased on second and third eggs. The full-filled line indicates predicted values from the linear mixed models and grey area correspond to ± SE values. Dots are the values of lipids per egg ($N_{first\ eggs}$=15, $N_{second\ eggs}$=17, $N_{third\ eggs}$=17).

❖ Yolk carotenoids

The absolute level of carotenoids varied between 11.61 μg and 67.23 μg and the concentration of carotenoids varied between 19.80 μg/g and 118.21 μg/g. Both absolute and concentration of carotenoids decreased significantly within the laying sequence (P<0.001; Table 7; Figure 8, Figure S1). Group size was not significant in the minimal models (linear and quadratic effect P>0.403; Table 7). Mother's condition, min. age and laying date were not significant and were not considered in model selection (all P>0.287). The concentration of carotenoids is dependent of the proteins, yolk, albumen and egg mass (Table S2).

Table 7. Factors affecting the absolute (a) and relative (b) level of yolk carotenoids

Explanatory terms	F	P	Estimates	SE	*df*
a) Absolute carotenoids					
Intercept			**40.604**	**2.433**	**50**
Laying order	**35.242**	**<.001**			
2			**-11.569**	**1.826**	**50**
3			**-14.949**	**1.873**	**50**
Group size	0.748	0.403			
Group size^2	0.086	0.774			
b) Concentration carotenoids					
Intercept			**67.912**	**5.088**	**50**
Laying order	**32.332**	**<.001**			
2			**-18.869**	**3.274**	**50**
3			**-26.087**	**3.355**	**50**
Group size	0.012	0.916			
Group size^2	0.0002	0.989			

Estimates and SE are given for explanatory terms included in the minimal model (bold characters)

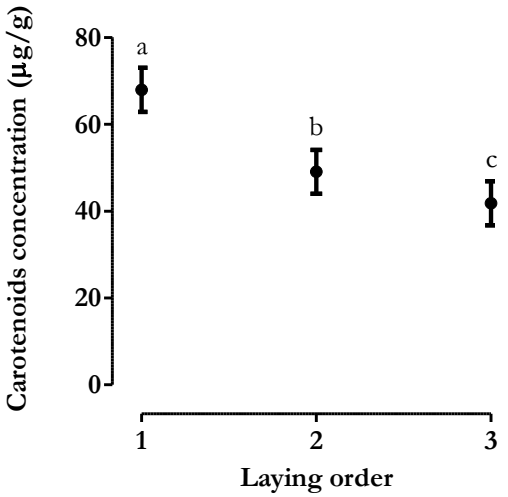

Figure 8. The concentration of carotenoids according to laying order. The data presented are predicted means±SE.

❖ Yolk hormones

▪ Yolk corticosterone

The absolute level of corticosterone changed between 14.067 pg and 42.488 pg and the concentration changed between 22.693 pg/mg and 60.516 pg/mg. Absolute and relative corticosterone increased significantly within laying sequence ($P<0.009$; Table 8a-b; Figure 9, Figure S4), the third eggs having higher levels of corticosterone. The number of helpers had no effect on absolute and relative levels of corticosterone (linear and quadratic effect $P>0.193$; Table 8a-b). Absolute and relative amounts of corticosterone decreased significantly with laying date (absolute level: estimate$=-0.488\pm0.221$, $F=4.980$, $P=0.037$; relative level: estimate$=-0.698\pm0.338$, $F=4.374$, $P=0.049$; Table 8a-b; Figure S2, Figure S3). Mother's condition and minimum age were not significant and did not enter in model selection (all $P>0.093$).

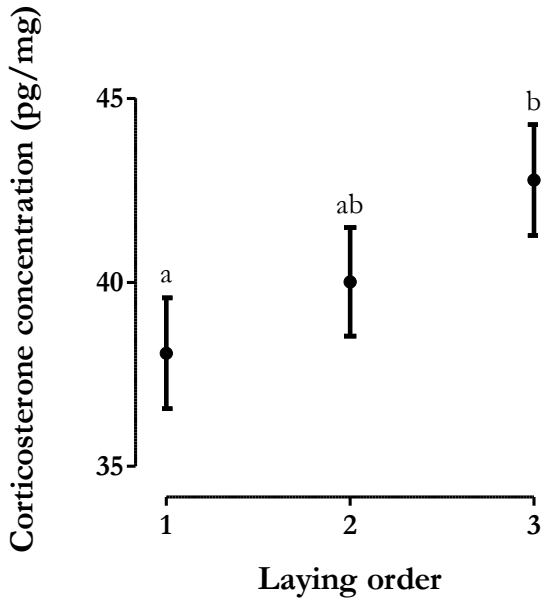

Figure 9. The concentration of corticosterone variation within laying sequence. The data presented are predicted means±SE.

- **Yolk androgens**

Testosterone

The absolute level of testosterone varied between 0.837 pg and 5.103 pg and the concentration of testosterone varied between 1.366 pg/mg and 6.731 pg/mg. Absolute (P=0.013) and relative (P=0.008) levels of testosterone decreased significantly along laying sequence, first eggs having higher testosterone concentration (Table 8c-d, Figure 10, Figure S5). Linear and quadratic effects of the number of helpers were not significant in both relative and absolute amounts of testosterone (all P>0.198; Table 8c-d). Mother's condition, min. age and laying date were not significant and hence did not enter in the model selection (all P>0.097). Concentration of testosterone was dependent of corticosterone, A4 and proteins (all P<0.046; Table S2).

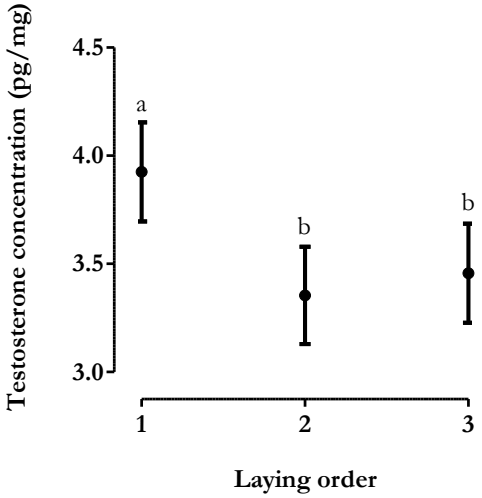

Figure 10. Testosterone concentration according to laying order. The data presented are predicted means±SE.

A4

The absolute level of A4 ranged between 3.613 pg and 11.619 pg and the concentration ranged between 6.163 pg/mg and 17.942 pg/mg. The absolute level of A4 decreased within the laying order (F=7.685, P=0.001; Table 8e), the first eggs had more quantity of this hormone than second and third eggs (Figure 12). Number of helpers did not have a significant effect on the absolute amount of A4 (linear and quadratic effect P>0.202; Table 8e), but the concentration of A4 tended to decrease linearly with group size (estimate=−0.448±0.223, F=4.165, P=0.064; Table 8f; Figure 11). Laying order had no significant effect on A4 concentration (P=0.098; Table 8f). Mother's condition, minimum age and laying date were not significant and hence did not enter in model selection (all P>0.207). A4 concentration was dependent of carotenoids and proteins (P<0.004; Table S2).

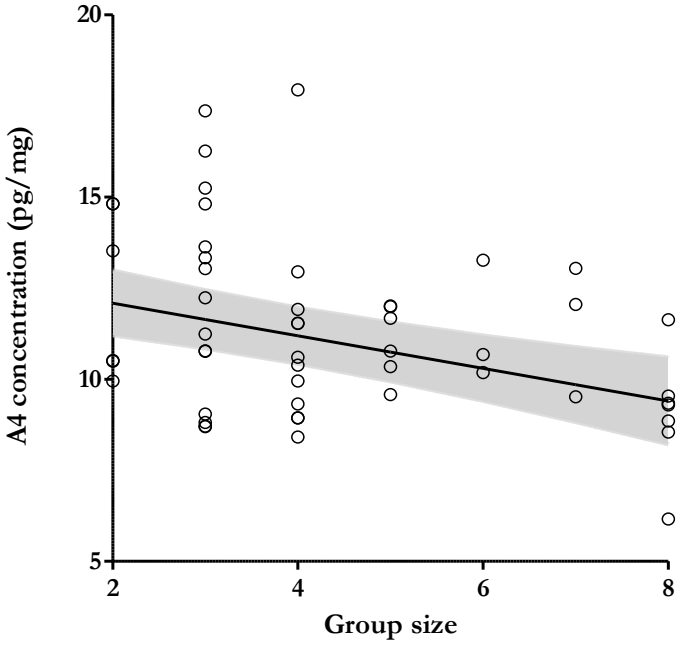

Figure 11. A4 concentration linearly decreases with breeding group size. The full-filled line indicates predicted values from the linear mixed models and grey area correspond to ± SE values. Dots are the values of A4 concentration per egg (N=52).

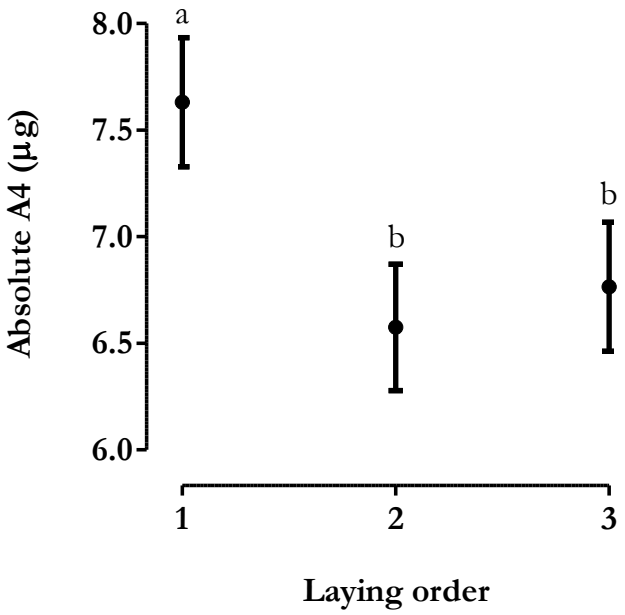

Figure 12. Absolute A4 according to laying order. The data presented are predicted means±SE.

Table 8. Factors affecting the absolute (a,c,e) and relative (b,d,f) level of yolk hormones: (a-b) corticosterone; (c-d) testosterone and (e-f) A4.

Explanatory terms	F	P	Estimates	SE	*df*
a) Absolute corticosterone					
Intercept			**26.857**	**0.184**	**50**
Laying date	**4.980**	**0.037**	**-0.488**	**0.221**	**21**
Laying order	**5.462**	**0.007**			
2			**0.853**	**1.079**	**50**
3			**3.510**	**1.105**	**50**
Group size	0.024	0.878			
Group size^2	0.134	0.722			
Mother's min. age	0.070	0.794			
b) Concentration corticosterone					
Intercept			**43.189**	**2.766**	**50**
Laying date	**4.374**	**0.049**	**-0.698**	**0.338**	**21**
Laying order	**5.157**	**0.009**			
2			**1.943**	**1.430**	**50**
3			**4.709**	**1.466**	**50**
Group size	1.904	0.193			
Group size^2	0.020	0.891			
c) Absolute testosterone					
Intercept			**2.419**	**0.153**	**50**
Laying order	**4.789**	**0.013**			
2			**-0.390**	**0.128**	**50**
3			**-0.256**	**0.131**	**50**
Group size	0.196	0.665			
Group size^2	1.856	0.198			
d) Concentration testosterone					
Intercept			**3.926**	**0.229**	**50**
Laying order	**5.385**	**0.008**			
2			**-0.571**	**0.184**	**50**
3			**-0.469**	**0.189**	**50**
Group size	0.050	0.826			
Group size^2	1.403	0.259			
e) Absolute A4					
Intercept			**7.631**	**0.303**	**50**
Laying order	**7.685**	**0.001**			
2			**-1.056**	**0.284**	**50**
3			**-0.866**	**0.291**	**50**
Group size	0.273	0.610			
Group size^2	1.823	0.202			
f) Concentration A4					
Intercept			**12.986**	**1.168**	**34**
Group size	**4.165**	**0.064**	**-0.448**	**0.223**	**12**
Laying order	2.501	0.098			
Group size^2	0.035	0.856			

Estimates and SE are given for explanatory terms included in the minimal model (bold characters)

Discussion

The aim of this study was to test if the egg mass and egg composition are affected by the number of helpers according to the laying order in a cooperatively breeding species. Group size is expected to improve rearing conditions and facilitate the survival of the whole clutch. Therefore, I expected that group size would influence egg traits affecting size hierarchies between siblings. No effect of group size was found on carotenoids and androgens, but I found that females might favour the survival of the first chicks through deposition of higher levels of carotenoids and androgens on first-laid eggs and corticosterone on last-laid eggs. By contrast, females breeding in large groups laid larger eggs with higher proteins levels and may mitigate size hierarchies within siblings through deposition of more lipids on later-laid eggs. These are correlative results and therefore, may not reflect causality. Nonetheless, these results suggest that group size and laying order interact to influence maternal allocation within clutches and this, in turn, may affect size hierarchy within broods.

Maternal investment in eggs

I found a positive quadratic effect of group size according to laying order on egg mass. Load-lightening of egg mass occurs until six individuals (breeding pair and four helpers) are present in the group, although it increases in groups composed by more than six individuals (particularly on second eggs). In addition to previous studies on cooperatively breeding species[26,75–77], this result clearly suggests that mothers adjust their investment in eggs in relation to the presence and number of helpers.

The previous work on sociable weaver showed a linear decrease effect of group size, instead of, a quadratic effect on egg mass[27] but this is probably due to the different range of group size included in the analysis. Since groups included on the previous study were up to six individuals (rather than 10 individuals), only the load-lightening on egg mass was detected. According to the life-history theory, females breeding in groups composed by up to four helpers may decrease the costs of producing eggs by reducing their mass and save energy or resources for future breeding attempts and own survival[1]. In fact, in superb fairy-wrens, in which females produce lighter eggs when breeding with helpers[26], females were found to have a greater recapture rate in the presence of helpers than males[106]. Similarly, a recent study in sociable weavers showed that females had also a higher survival probability when breeding with more helpers than males[86]. The positive effect of helpers on female survival was only detectable in young females (or those who immigrated from other colonies)[86], although mother's minimum age had no effect on egg mass. The female condition was expected to be positively correlated with egg mass[107], although it did not affect egg mass.

Females breeding in large groups (with more than four helpers) may improve their investment on heavier eggs as the costs of rearing bigger chicks (with higher demands) are only affordable when raised in big groups[108]. Females may be able to do this higher investment because groups which have more than four helpers provide most of the help during food provisioning and consequently, they may reduce the mother's costs at this stage.

❖ Maternal allocation

According to the energetic model of Audouin Gull[34], females were expected to save energy by reducing the amount of albumen for the first egg and yolk deposition for second and third eggs. Differently from expected, albumen mass increased with group size on second eggs which may cause the pronounced increase of egg mass. Therefore second eggs may be better provisioned with water and proteins[109]. The quality of the mothers may also determine the maternal allocation although body condition only showed a tendency to affect albumen mass. Yolk mass increased with group size independently of laying order but, in a previous study, yolk mass was showed to decrease with laying order[78], thus more data is needed to clarify the allocation of this component. No quadratic effect was detected on egg components analysis probably because the difference of sample size between egg mass (N=101 clutches) and egg components (N=18 clutches).

According to previous studies[26,27], females were expected to take advantage of the number of helpers and reduce the allocation of costly nutrients and hormones. However, both the yolk mass and the levels of yolk proteins increased with group size. The increase of egg mass on large groups within the clutch may be due to the increase of yolk proteins (and consequently yolk mass) with group size. This result suggests that mothers invest more energy on nutrients that are essential during embryonic development when breeding in large groups. In addition, androgens affect begging rate (Table 1) and were expected to decrease with group size as the number of helpers increase food provisioning and may reduce the need of begging[110]. Although, testosterone did not vary in relation to group size and A4 concentration tended to decrease with group size, but more data is needed to confirm the influence of group size on A4 concentration.

Females breeders were expected to have a brood reduction strategy in which first-laid eggs would be favoured in relation to remaining eggs of the clutch. As a result, costly nutrients should be preferentially invested in first-laid eggs because they have a higher chance of survival[78] and are expected to be less costly to produce[34]. In accordance with this hypothesis, first eggs contained higher levels of carotenoids. If first eggs have more carotenoids, then first-hatching chicks will have the highest immune functions and the strongest gape coloration within the brood (Table 1) which may positively influence the amount of food they will receive during nestling and increase their chance of survival[111].

Later-laid eggs are expected to be more costly to produce[34] and this could entail higher stress levels on females. As expected, higher levels of corticosterone were found on later-laid eggs. This hormone is transmitted passively to later-laid eggs and it may negatively affects the hatching size, growth and survival[20] (Table 1) of the chicks hatching from these eggs. Yolk corticosterone was also negatively affected by laying date, indicating a decrease of stress throughout the breeding season (at least until 28th of September).

Proteins may be dependent of carotenoids' deposition into eggs (Table S2). In opposition to carotenoids, proteins increased with the laying sequence and may mitigate some of the negative effects of hormones and carotenoids on later-laid eggs.

Androgens (testosterone and A4) decreased with laying order, potentially causing negative effects on offspring's growth, competitiveness and immune responses on later-laid eggs (Table 1).

These results suggest that levels of hormones (corticosterone and androgens) and carotenoids may exacerbate size hierarchies between siblings possibly facilitating brood reduction while proteins may mitigate some of the detrimental effects of these components independently of group size.

I found that only the allocation of lipids, but not androgens, varied with group size according to laying order. Nonetheless, as big groups provide a better rearing condition, females may mitigate brood hierarchies and compensate for the detrimental effects caused by hormones (lower levels of androgens and higher levels of corticosterone) and lower levels of carotenoids on later-laid eggs through deposition of higher levels of lipids in later eggs. Lipids provide most of the energy during growth and development[112] and the higher investment of lipids on later-laid eggs may increase the survival of these eggs.

Small groups may not support the rearing conditions needed for the whole brood to survive and in this case, in addition to the levels of carotenoids and hormones, females may exacerbate hierarchical differences through the higher investment of lipids on the first-laid eggs and lower investment on later-laid eggs.

This is a correlative study and an experimental manipulation of the number of helpers before laying remain to be done to fully test the causal effect of helpers on maternal investment and allocation. I cannot completely exclude the confounding effects of colony size in my results and the presence/absence of protection against snakes in egg composition analysis. However, both colony size and protection of colonies are unlikely to explain the results presented here. First, colony size is positively correlated with group size (Spearman Rank Correlation: $R=0.57$, $P=0.011$) and therefore, would be expected to affect androgens in the same direction than group size. However in a previous study, androgen concentration increased with colony size[78] instead of decreased as found in relation to group size. The protection against snakes may affect female reproductive strategies and therefore, affect maternal allocation. If females change their investment in eggs according to the protection of the colonies, it was expected that females should invest more in eggs from protected colonies than non-protected colonies as these eggs have higher chances to survive. The protection did not have an effect on egg mass, but its effect on egg composition remains to be tested. It was also expected that age of the females could be a confounding effect as younger breeders have fewer helpers than older breeders. However, no correlation was found between females' minimum age and group size (Spearman Rank Correlation: $R=0.14$, $P=0.316$).

Conclusion

This is the first study analysing how group size affects female investment and allocation of yolk contents depending on laying order. I found that group size differently affects egg mass, albumen mass and yolk lipids depending on the egg order in the laying sequence. Therefore, it is essential to consider laying order when investigating the effect of

group size on female investment in cooperatively breeding species as group size could alter hierarchical differences within the brood. Further studies are needed to see if the maternal allocation affects hierarchical differences within the brood in terms of growth and survival according to the group size. Proteins and carotenoids may be dependently deposited in the eggs and vary in an opposite way with the laying sequence and therefore, it is important to consider both components to fully understand maternal allocation strategies in this species.

Maternal reproductive strategies are essential to understanding the adaptive significance of helping behaviour in cooperative breeding systems, as helpers potentially increase mother's reproductive success, affecting maternal investment and allocation.

Females may take advantage of the number of helpers by adjusting the investment in the current reproduction according to the future help available and food availability. When the amount of help provided by the group and/or food availability are not enough to rear a complete clutch, females may benefit by increasing the quality on fewer chicks that will probably survive and thereby save energy for survival and future reproduction. The opposite scenario occurs when females invest more in the current reproduction when having high availability of food because the helpers afford most of the help and, consequently females might be able to save energy during food provisioning and favour survival of complete clutches. These strategies may allow females to use their energy and resources in a more efficient way to maximize their lifetime reproductive success. Therefore, future study of females and offspring fitness should help to understand the evolutionary consequences of maternal allocation.

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Supplementary material

Appendix 1. Egg content analysis

Carotenoids, proteins and lipids concentrations were analysed at Centre d'Ecologie Fonctionnelle & Evolutive and hormonal analysis at Centre d'Etudes Biologiques de Chizé.

❖ Carotenoids

Carotenoids concentration was determined by colorimetry [98,99].

For carotenoid extraction 60 mg of egg yolk was diluted with acetone (1µg of acetone for 0.1mg of yolk). Sample were vortexed, kept overnight at -20°C and then centrifugated (10 minutes at 13000g, at 4ºC). We extracted 125 µl of supernatant for each sample and determined the optic density (OD) at 450nm in a microplate photometer (Victor^3 1420 Multilabel Plate Reader, Perkin-Elmer). Commercial solution of lutein (xanthophylls Sigma X-6250) was use to realize a serial dilution and obtain a standard curve to determine the relationship between the OD value and carotenoid concentration in yolk eggs. Inter- and intra-samples variations were respectively 17.55% and 2.61%. We used the mean of the two closest values obtained for the three replicates as the carotenoid concentration in fresh yolk eggs.

❖ Corticosterone, Testosterone and A4

Hormones were quantify by radioimmunoassay, based on the antigen-antibody reaction [100].

100 mg of each sample were homogenised in 1 mL of distilled water. Steroids were extracted by adding 3 mL of diethyl-ether to 300 µL of the mixture, vortexing and centrifuging (5 minutes at 2000 rpm, at 4°C). The diethyl-ether phase containing steroids was decanted and poured off after snap freezing the tube in an alcohol bath at 38°C. This was done twice for each yolk and the resultant was then evaporated. The dried extracts were redissolved in 900 µL of phosphate buffer and each hormone was assayed in duplicate. 100 µL of extract were incubated overnight with 4000 cpm of the appropriate 3H-steroid (Perkin Elmer, US) and polyclonal rabbit antiserum. Corticosterone assays used the antibody "anti-11-HS-corticosterone" supplied by P.A.R.IS. (France), testosterone used the antibody "Anti-testosterone" provided by Dr. Picaper (médecin nucléaire, CHU La Source, Orléans, France) and A4 used "anti-androstenedione" by Sigma (US). The bound fraction was then separated from free fraction by addition of dextran-coated charcoal and activity was counted on a tri-carb 2810 TR scintillation counter (Perkin Elmer, US). Cross-reactions of corticosterone antiserum were as follows: cortisone (53 %), 20α-hydroxyprogesterone (2.5 %), cortisol (2 %), progesterone (1.3 %), Δ4-pregnen-21-ol-3,20-dione (0.5 %), aldosterone (0.2 %), 17α-hydroprogesterone (0.1%), 1,3,5(10)-estratrien-3,17-diol (0.1 %), 20β-hydroxyprogesterone

(<0.03 %). Inter- and intra-assay variations were respectively 30.14% and 7.07% for corticosterone, 18.01% and 7.27% for testosterone, 20.38% and 4.76% for A4. Tests were performed to validate the three hormones assays on egg yolk samples. Tests were performed to validate the hormone assays on egg yolk samples: inter- and intra-assay variations were respectively 18.01% and 7.27% for testosterone, 20.38% and 4.76% for androstenedione, 30.14% and 7.07% for corticosterone. Testosterone, androstenedione and corticosterone lowest detectable concentrations were respectively 1.59 pg/mg, 2.07 pg/mg and 1.55 pg/mg. Two yolk samples were serially diluted in the assay buffer and their displacement curves were parallel to the standard curve.

❖ Proteins

About 2 mg of dried yolk was weighed in a microbalance (Sartorius MC5) in a tin capsule, sealed and placed in an auto sampler, from which it was dropped into a combustion chamber. As the sample entered, the combustion chamber oxygen was injected into the carrier gas (He), which flowed through the combustion tube. The temperature raised up to 1800 °C, which insured complete combustion of the sample. Inter and intra variations were 5.10% and 2.34%, respectively.

❖ Lipids

180 mg of dried yolk was introduced into tubes resistant to the chloroform (type Sarstedt 15 ml). 3 ml of deionized (DI) water, 6 ml of methanol and 3 ml of chloroform were added to the samples. After, the samples were vortexed (30 seconds at 2400 rpm), 3 ml of DI water and 3 ml of chloroform were added and then, vortexed and centrifuged (10 minutes at 4500 rpm, 2649g). 8. Samples were biphasic, the water and methanol were positioned on the top, the chloroform and lipids on the bottom and one thin layer of proteins divided the two phases. The chloroform and lipids were extracted with a Pasteur pipette into a glass tube previously weighted and reserved. 3 ml of chloroform were added again in the sample, vortexed and centrifuged again. We did a second extraction of the chloroform and added it with the previous extraction. The tubes with the extractions were put in heating plates (60ºC) and when all chloroform evaporated, tubes containing the lipids were weighted. Mass of lipids is given by the difference between the weight of the tubes containing the lipids and the weight of empty tubes. Overall variation of 32.34%.

Appendix 2. Summary table of the sample size, colony size, protection and pit-tags

Table S1. Summary table of the sample size, colony size, protection and existence of pit-tags from the 13 colonies included in this study.

Colony	Egg mass analysis	Egg constituents analysis		Colony size	Protected/non-protected	Pit-tags
	Number of clutches considering group size and laying order	Number of clutches without group size	Number of clutches considering group size			
2	0	0	0	21	Non-protected	No
7	0	2	0	14	Non-protected	No
8	33	11	9	104	Protected	No
11	9	-	-	44	Protected	No
20	5	-	-	30	Protected	No
21	1	-	-	7	Non-protected	No
27	2	-	-	25	Protected	Yes
31	9	4	3	45	Protected	Yes
32	6	2	1	21	Non-protected	No
37	25	8	4	86	Protected	Yes
38	6	1	1	17	Non-protected	No
71	3	-	-	31	Protected	Yes
81	2	-	-	19	Non-protected	No
Total sample	101	28	18			

Appendix 3. Relation between egg contents

Table S2. Relations between the concentration of yolk contents, yolk mass, albumen mass and egg mass. Estimates ± SE (p-values).

Dependent variable / Independent variable	Testosterone (pg/mg)	Corticosterone (pg/mg)	A4 (pg/mg)	Carotenoids (µg/g)
Corticosterone (pg/mg)	**0.034 ± 0.015 (p=0.028)**			
A4 (pg/mg)	**0.258 ± 0.041 (p<0.001)**	-0.523 ± 0.311 (p=0.103)		
Carotenoids (µg/g)	0.008 ± 0.005 (p=0.147)	-0.057 ± 0.034 (p=0.109)	**0.032 ± 0.011 (p=0.004)**	
Proteins (%)	**-0.102 ± 0.050 (p=0.046)**	-0.215 ± 0.341 (p=0.529)	-0.078 ± 0.107 (p=0.472)	**-2.311 ± 0.963 (p=0.021)**
Lipids (%)	0.006 ± 0.007 (p=0.336)	0.050 ± 0.044 (p=0.258)	0.008 ± 0.014 (p=0.579)	0.214 ± 0.126 (p=0.097)
Yolk mass (g)	-1.209 ± 1.565 (p=0.460)	13.697 ± 9.965 (p=0.179)	-1.875 ± 3.184 (p=0.558)	**-57.453 + 26.625 (p=0.033)**
Albumen mass (g)	-0.561 ± 0.669 (p=0.408)	1.298 ± 4.272 (p=0.778)	-0.304 ± 1.358 (p=0.838)	**-33.465 + 11.246 (p=0.004)**
Egg mass (g)	-0.890 ± 0.633 (p=0.170)	5.369 ± 4.026 (p=0.204)	-1.590 ± 1.276 (p=0.229)	**-48.038 ± 10.110 (p<0.001)**
Dependent variable / Independent variable	**Proteins (%)**	**Lipids (%)**	**Yolk mass (g)**	**Albumen mass (g)**
Lipids (%)	-0.014 ± 0.013 (p=0.266)			
Yolk mass (g)	3.373 ± 2.512 (p=0.186)	-29.020 ± 20.944 (p=0.172)		
Albumen mass (g)	-0.903 ± 1.076 (p=0.387)	8.793 ± 8.620 (p=0.276)	-0.057 ± 0.042 (p=0.182)	
Egg mass (g)	-0.240 ± 0.985 (p=0.781)	1.654 ± 8.108 (p=0.653)	**0.121 ± 0.038 (p=0.002)**	**0.800 ± 0.046 (p<0.001)**

Appendix 4. Laying order and laying date effects on carotenoids, hormones and proteins

❖ Yolk carotenoids

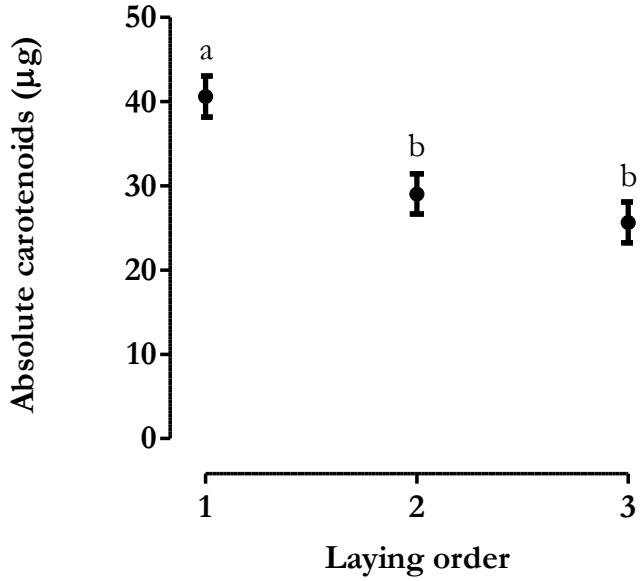

Figure S1. Absolute carotenoids according to laying order. Data presented are predicted means±SE.

❖ Yolk hormones

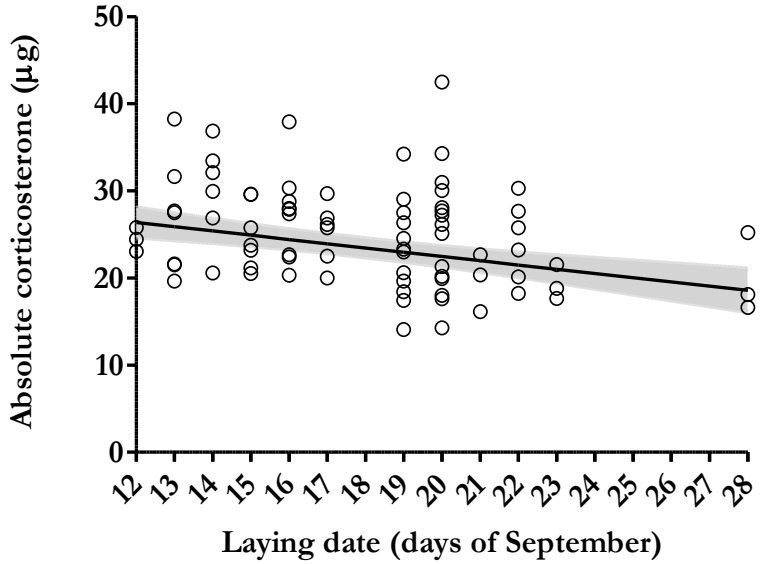

Figure S2. Linear relationship between absolute corticosterone and laying date. The line indicates the predicted values. Dots correspond to real data of absolute corticosterone per egg (N=80).

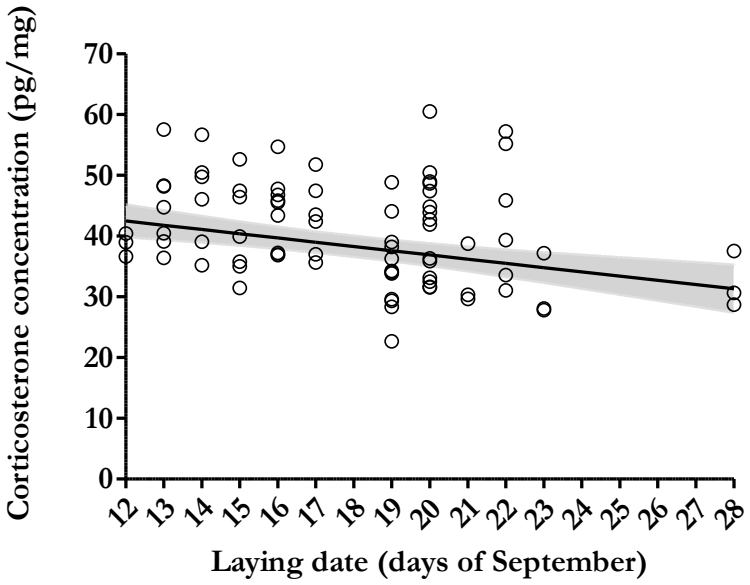

Figure S3. Linear relationship between corticosterone concentration and laying date. The line indicates the predicted values. Dots correspond to real data of corticosterone concentration per egg (N=80).

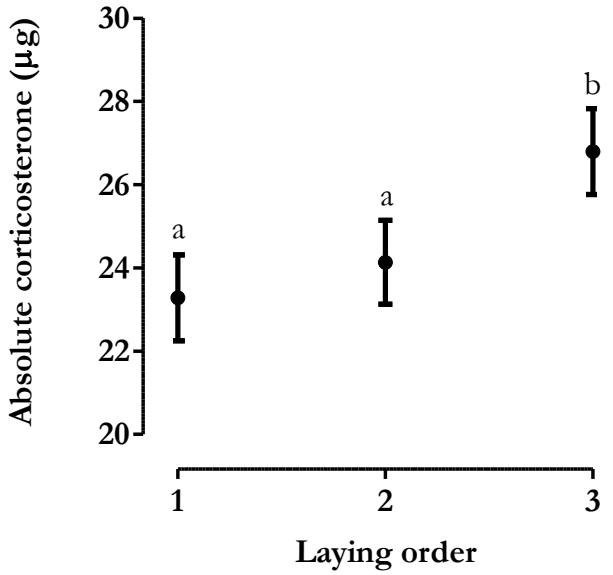

Figure S4. Absolute corticosterone according to laying order. Data presented are predicted means±SE.

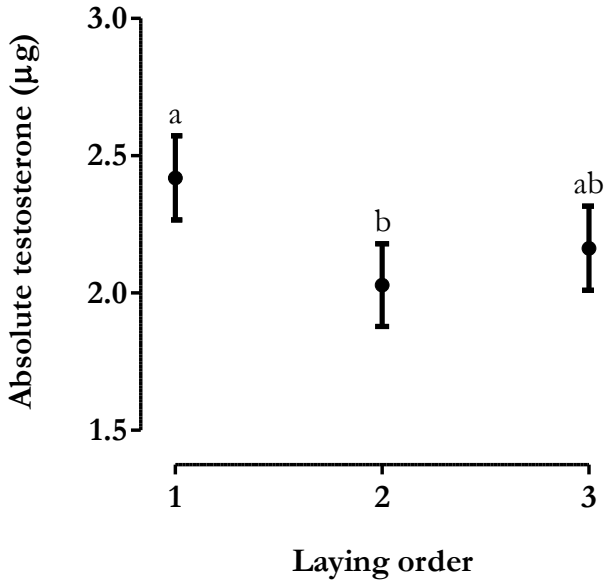

Figure S5. Absolute testosterone according to laying order. Data presented are predicted means±SE.